FORM PTO-1390 (REV. 1-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	1422-411P
U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	09/485298 NEW
INTERNATIONAL APPLICATION NO.	PCT/JP98/03566
INTERNATIONAL FILING DATE	August 10, 1998
PRIORITY DATE CLAIMED	August 14, 1997
TITLE OF INVENTION	METHODS FOR DNA AMPLIFICATION AND KITS THEREFOR
APPLICANT(S) FOR DO/EO/US	YAMAMOTO, Junko; MUKAI, Hiroyuki; HINO, Fumitsugu; KATO, Ikunoshin

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(3)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98./-1449 W/ 8 References and International Search Report (PCT/ISA/210)
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment and Sequence Listing W/ Disk (7 total sheets).
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
    1.) Zero (0) sheets of Formal Drawings

420 Rec'd PCT/PTO 0 8 FEB 2000

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/485298	PCT/JP98/03566	1422-411P

17. ☒ The following fees are submitted:

	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. **$970.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$840.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO. **$690.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4). **$96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	18 - 20 =	0	X **$18.00**	$	0
Independent Claims	4 - 3 =	1	X **$78.00**	$	78.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)		Yes	+ **$260.00**	$	260.00
TOTAL OF ABOVE CALCULATIONS =				$	1178.00
Reduction of ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$	1178.00
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$	1178.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	40.00
TOTAL FEES ENCLOSED =				$	1218.00
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ **1218.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account. No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2448.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Send all correspondence to:
Birch, Stewart, Kolasch & Birch, LLP or Customer No. 2292
P.O. Box 747
Falls Church, VA 22040-0747
(703)205-8000

SIGNATURE _____

WEINER, MARC S.
NAME

#32,181 (MSW)
REGISTRATION NUMBER

/dll February 8, 2000

IN THE U.S. PATENT AND TRADEMARK OFFICE

APPLICANTS: YAMAMOTO, Junko et al

INT'L. APPLN. NO.: PCT/JP98/03566

SERIAL NO.: NEW GROUP:

FILED: February 8, 2000 EXAMINER:

FOR: METHODS FOR DNA AMPLIFICATION AND KITS THEREFOR

PRELIMINARY AMENDMENT

BOX PATENT APPLICATION
Assistant Commissioner of Patents
and Trademarks
Washington, D.C. 20231

February 8, 2000

Sir:

The following Preliminary Amendments and Remarks are respectfully submitted in connection with the above-identified application.

IN THE SPECIFICATION:

Before line 1, insert --This application is the national phase under 35 U.S.C. §371 of PCT International Application No. PCT/JP98/03566 which has an International filing date of August 10, 1998 which designated the United States of America.--

Please delete the pages following the abstract containing the Sequence Listing. Please insert the substitute Sequence Listing enclosed herewith immediately after the abstract.

IN THE CLAIMS:

CLAIM 4: Lines 1 and 2, change "any one of claims 1 to 3" to --claim 1--

CLAIM 6: Lines 1 and 2, change "any one of claims 1 to 5" to --claim 1--

CLAIM 7: Lines 1 and 2, change "any one of claims 1 to 6" to --claim 1--

CLAIM 8: Lines 1 and 2, change "any one of claims 1 to 7" to --claim 1--

CLAIM 9: Lines 1 and 2, change "any one of claims 1 to 8" to --claim 1--

CLAIM 13: Line 1, change "any one of claims 10 to 12" to --claim 10--

CLAIM 14: Line 1, change "any one of claims 10 to 13" to --claim 10--

CLAIM 15: Line 1, change "any one of claims 10 to 14" to --claim 10--

CLAIM 16: Line 1, change "any one of claims 10 to 15" to --claim 10--

REMARKS

The specification has been amended to provide a cross-reference to the previously filed International Application.

The above amendment to the claims is merely to delete the undesired multiple dependencies and to place the application into better form prior to examination.

Enclosed herewith in full compliance with 37 C.F.R. §§1.821-1.825 is a substitute Sequence Listing to be inserted into the specification as indicated above. The substitute Sequence Listing in no way introduces new matter into the specification. Also submitted

herewith in full compliance with 37 C.F.R. §§1.821-1.825 is a disk containing the computer readable form of the substitute Sequence Listing. The computer readable form of the Substitute Sequence Listing, file "1422-411.app", is identical to the paper copy, except that it lacks formatting.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §1.16 or under 37 C.F.R. §1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____

MARC S. WEINER
Reg. No. 32,181
P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

MSW/JDK/dll

Attachments: Disk Containing the Computer Readable Form of Substitute Sequence Listing, Paper Copy of Substitute Sequence Listing

DESCRIPTION

METHODS FOR DNA AMPLIFICATION AND KITS THEREFOR

TECHNICAL FIELD

The present invention relates to a method for amplifying DNA, particularly to RT-PCR method capable of selectively amplifying only a nucleotide sequence corresponding to RNA even in admixture of DNAs, and to a kit usable for the method for amplifying DNA.

BACKGROUND ART

A method for amplifying DNA, particularly polymerase chain reaction (PCR) method, is a technique for simply amplifying a desired nucleic acid fragment *in vitro*, and presently has been an indispensable experimental method in a wide variety of fields of biology, medicine, agriculture, and the like, as well as in the studies relating to genes. PCR method is originally a technique for amplifying DNA, and there has been developed PCR method for amplifying a DNA fragment having a nucleotide sequence corresponding to RNA, which is so-called RT-PCR method. The method is a method for specifically amplifying cDNA derived from RNA, comprising synthesizing a DNA transcriptional product which is complementary to RNA (cDNA) by using a reverse

transcriptase having a RNA-dependent DNA polymerase activity, namely a reverse transcription activity, wherein the reverse transcriptase is a kind of a DNA polymerase, or using a DNA polymerase also having a reverse transcription activity, and subsequently carrying out PCR by the use of the resulting transcription product as a template. Aside from the fact that RT-PCR method is utilizable for cloning of cDNA derived from mRNA and for preparation of a cDNA library, it is also useful as a method for examining an expression state of a particular mRNA.

However, when DNA is admixed in an RNA sample to be used in RT-PCR, since products in which a certain region on DNA encoding RNA and a pseudogene are used as templates for PCR are simultaneously amplified, it would be difficult to selectively obtain only the amplified product by the use of RNA as a template. In order to prevent the formation of an amplified product derived from DNA admixed in such an RNA sample, it would be necessary to use a purified RNA as a sample, or to use a sample in which an admixed DNA is removed by DNA degradation enzyme treatment, or the like, thereby making its procedures complicated.

As a method for solving this problem, there has been proposed a method for selectively amplifying a DNA fragment derived from cDNA which is complementary to RNA,

comprising synthesizing an RNA-cDNA double-stranded nucleic acid of which *Tm* value, i.e. melting temperature, is low, by the reverse transcription reaction in the presence of a nucleotide analog dITP, and further carrying out PCR reaction at a low-temperature of denaturation temperature in the presence of the same nucleotide analog, thereby suppressing the amplification of a product caused by DNA admixed in a sample [*Nucl. Acids Res.* **24**, 5021-5025 (1996)]. However, this method needs to change the amount of dITP and the reaction temperature depending upon a GC content of an amplified nucleotide sequence, thereby making it difficult to find the optimal conditions. Further, it is not satisfactory in its reactivities, and in some cases selective amplification does not take place depending on a target nucleotide sequence.

DISCLOSURE OF INVENTION

In view of overcoming the defects in the prior art, an object of the present invention is to provide a method for amplifying DNA capable of selectively obtaining only a DNA fragment having the nucleotide sequence corresponding to RNA even in admixture of DNA, the method having excellent wide utility, reproducibility and detection sensitivity.

The present inventors have elucidated that one of the

problems of the method using the above nucleotide analog resides in that it has been difficult to set temperature conditions such that an RNA-cDNA double-stranded nucleic acid comprising a template RNA and cDNA to which a nucleotide analog is incorporated is denatured, and that a usual double-stranded DNA is not denatured. Moreover, they have found that this problem can be solved by adding a compound for lowering *Tm* value of an RNA-cDNA double-stranded nucleic acid during DNA amplification reaction, the compound including, for instance, formamide, and the like.

Further, the present inventors have considered that another problem of the above method is that the incorporation of a nucleotide analog into the DNA strand synthesized in the above method is affected by a nucleotide sequence of a target RNA, particularly by its GC content. The present inventors have found that DNA amplification takes place with excellent reproducibility regardless of the GC content of a target RNA by adding to the reaction mixture two or more kinds of nucleotide analogs to be incorporated into DNA at a given frequency, and the present invention has been completed thereby.

In sum, the present invention is concerned with:

[1] a method for amplifying a DNA by the use of a DNA fragment comprising a nucleotide analog as a template in

the presence of nucleotide analogs, characterized in that the method for amplifying a DNA is carried out in the presence of two or more kinds of nucleotide analogs or in the presence of a compound for lowering *Tm* value of a double-stranded nucleic acid;

[2] a method for amplifying a DNA by the use of a DNA fragment comprising a nucleotide analog as a template in the presence of nucleotide analogs, characterized in that the method for amplifying a DNA is carried out in the presence of one or more kinds of nucleotide analogs and a compound for lowering *Tm* value of a double-stranded nucleic acid;

[3] a kit for amplifying a DNA in the presence of a nucleotide analog by the use of a DNA fragment comprising a nucleotide analog as a template, characterized in that the kit comprises two or more kinds of nucleotide analogs or a compound for lowering *Tm* value of a double-stranded nucleic acid; and

[4] a kit for amplifying a DNA in the presence of a nucleotide analog by the use of a DNA fragment comprising a nucleotide analog as a template, characterized in that the kit comprises one or more kinds of nucleotide analogs and a compound for lowering *Tm* value of a double-stranded nucleic acid.

BEST MODE FOR CARRYING OUT THE INVENTION

One of the great features of the method for amplifying a DNA of the present invention resides in that, in the method for amplifying a DNA by the use of a DNA fragment comprising a nucleotide analog as a template, the method being carried out in the presence of nucleotide analogs, the method for amplifying DNA is carried out in the presence of two or more kinds of nucleotide analogs and/or in the presence of a compound for lowering *Tm* value of a double-stranded nucleic acid.

In the method of the present invention, as a DNA fragment to be used as a template for amplification reaction, there can be used cDNA prepared by reverse transcription reaction by the use of RNA as a template in the presence of one or more kinds of nucleotide analogs. The reverse transcription reaction may be carried out in further presence of a compound for lowering *Tm* value of a double-stranded nucleic acid.

A sample comprising RNA to which the method of the present invention can be applied is not particularly limited. Examples thereof include biologically derived samples such as cells, tissues and blood, and samples having possibility of containing organisms, the samples including foods, soils and wastewater. In addition, a nucleic acid-containing preparation obtainable by treating

the above samples by a known method may be also used. As the preparations, there can be used in the present invention, for instance, samples obtainable from cell disrupted products or fractionated products thereof, samples rich in whole RNAs, or particular RNA molecules, for instance, mRNA in the samples, and the like. Incidentally, DNA corresponding to RNA can be selectively amplified in these samples without being affected from coexisting DNAs, even if DNAs were contained in these samples. In addition, there may be used samples from which DNA is removed by a known method.

The RNA to be amplified by the method of the present invention is not particularly limited. Examples thereof include an RNA molecule such as whole RNA, mRNA, tRNA and rRNA, or a particular RNA molecule, including a given RNA that can be used to prepare a primer used in reverse transcription and amplification reactions. For instance, as long as the presence or absence of the target RNA and the increase or decrease in its level indicate a particular disease, the diagnosis of the disease can be made by the method of the present invention. In addition, the presence of a microorganism in a sample can be detected by using the microorganism-specific RNA as a target. The used amount of RNA to be amplified in one reverse transcription reaction may be preferably an amount

sufficient for carrying out reverse transcription reaction. For instance, from the viewpoint of the sensitivity, the amount is preferably 0.1 μg or more, more preferably 0.5 μg or more, and from the viewpoint of the reaction inhibition, the amount is preferably 2 μg or less, more preferably 1 μg or less.

The primer used in cDNA synthesis from a target RNA in the present invention is an oligonucleotide having a nucleotide sequence complementary to the target RNA, and the primer is not particularly limited as long as it anneals against the target RNA in operable reaction conditions. The primer may be oligonucleotides such as oligo(dT) and oligonucleotide(random primer) having a random sequence.

The length of the primer is preferably 6 nucleotides or more, more preferably 10 nucleotides or more, from the viewpoint of carrying out specific annealing, and the length is preferably 100 nucleotides or less, more preferably 30 nucleotides or less, from the viewpoint of the oligonucleotide synthesis. The oligonucleotide can be synthesized by, for instance, phosphoamidite method using DNA Synthesizer Model 394 manufactured by ABI (Applied Biosystems Inc.). Besides the above, oligonucleotides synthesized by any of methods such as triphosphate method, H-phosphonate method and thiophosphite method may be used.

In addition, it may be biological sample-derived oligonucleotides, including, for instance, those prepared by isolating from a restriction endonuclease digest of DNAs prepared from the sample. The used amount of the primer in a reverse transcription reaction mixture may be an amount sufficient for carrying out cDNA synthesis from a target RNA. From the viewpoint of the synthesis efficiency, the amount is preferably 0.1 μM or more, more preferably 0.5 μM or more, and from the viewpoint of the reaction inhibition, the amount is preferably 10 μM or less, more preferably 5 μM or less.

In addition, the primer used in amplification reaction of DNA (PCR) is also not particularly limited, as long as the primer can be used in amplification of DNA by the use of cDNA corresponding to a target RNA as a template, and various oligonucleotides similar to ones mentioned above can be used. The primer used in the cDNA synthesis from the above target RNA may be used in the amplification reaction of DNA. The used amount of the above primer in a PCR mixture may be an amount sufficient for carrying out synthesis for double-stranded DNA by the use of a template cDNA obtained from a target RNA as a template. From the viewpoint of the synthesis efficiency, the amount is preferably 0.05 μM or more, more preferably 0.1 μM or more, and from the viewpoint of the reaction

specificity, the amount is preferably 2 μM or less, more preferably 1 μM or less.

The enzyme having a reverse transcription activity usable in the present invention is not particularly limited, as long as the enzyme has a cDNA synthesizing activity by the use of RNA as a template. For instance, there can be included reverse transcriptases of various origins, including avian myeloblastosis virus-derived reverse transcriptase (AMV-RTase), Moloney's mouse leukemia virus-derived reverse transcriptase (MMLV-RTase), Raus-associated virus 2-derived reverse transcriptase (RAV-2-RTase), and the like. Besides the above, DNA polymerases also having a reverse transcription activity can be used. For instance, there can be used *Thermus thermophilus*-derived DNA polymerase (Tth DNA polymerase), *Bacillus caldotenax*-derived DNA polymerase (Bca DNA polymerase), and the like. AMV-RTase or RAV-2-RTase is preferable. These enzymes may be any of those obtained by purifying from the natural origins, or recombinant proteins produced by genetic engineering means. The used amount of the enzyme having a reverse transcription activity may be an amount sufficient for carrying out reverse transcription reaction.

One of the great features of the method for amplifying a DNA of the present invention resides in that

DNA amplification reaction is carried out by the use of cDNA formed by the reverse transcription reaction as a template, thereby amplifying the DNA. As DNA amplification reaction, PCR method (Japanese Examined Patent Publication Nos. Hei 4-67957 and 4-67960) is preferably used.

As DNA polymerases usable in PCR method, various thermostable DNA polymerases can be used, including, for instance, *Thermus thermophilus*-derived DNA polymerase (Tth DNA polymerase), *Thermus aquaticus*-derived DNA polymerase (Taq DNA polymerase), *Pyrococcus furiosus*-derived DNA polymerase (Pfu DNA polymerase), and other thermostable DNA polymerases. These enzymes may be used alone or in admixture. These enzymes may be any of proteins obtained by purifying from the natural origins, or recombinant proteins produced by genetic engineering means.

In addition, when a DNA polymerase also having the reverse transcription activity mentioned above is used as a reverse transcriptase, since the same DNA polymerase can be reused also in PCR reaction, the reverse transcription reaction and the PCR reaction can be carried out by using a single reaction tube.

The amount of the DNA polymerase may be an amount usually employed when polymerase reaction is carried out.

The nucleotide analog described in the present

specification refers to a substance other than dATP, dCTP, dGTP or dTTP, wherein the substance is incorporated into cDNA freshly synthesized in cDNA synthesis reaction by the use of RNA as a template and in DNA amplification reaction by the use of the cDNA as a template, and does not cause to stop the reaction. Particularly, there can be used in the present invention nucleotide analogs having a property of lowering *Tm* value of DNA by incorporation of these nucleotide analogs. The nucleotide analog used in the present invention is not particularly limited, and there can be used 7-Deaza-dGTP, 7-Deaza-dATP, dITP, hydroxymethyl dUTP, and the like.

In cDNA synthesis and DNA amplification using one kind of a nucleotide analog, the incorporation frequency of a nucleotide analog may be affected depending upon the GC content of RNA to be targeted in some cases. For instance, dITP is incorporated in a DNA strand in place of dGTP, and there may be some cases where the higher the GC content of a target RNA, the more its incorporation frequency, even under the same reaction conditions. In the present invention, the conditions for selective amplification of a product corresponding to an RNA can be easily set by using two or more kinds of appropriate nucleotide analogs in combination, so that the combined incorporation frequency of these nucleotide analogs is not

be influenced by the GC content of the target RNA.

The combination of nucleotide analogs as described above is not particularly limited, and two or more kinds of nucleotide analogs which are likely to be incorporated uniformly can be used in appropriate combination. For instance, there can be used in the present invention a combination of a nucleotide analog to be incorporated in a DNA strand in place of dATP or dTTP with a nucleotide analog to be incorporated in a DNA strand in place of dCTP or dGTP. In particular, a combination of 7-Deaza-dATP and 7-Deaza-dGTP can be preferably used.

The content of a nucleotide analog in a reverse transcription reaction mixture is preferably 50 μM or more, more preferably 55 μM or more, from the viewpoint of sufficiently exhibiting an effect of lowering *Tm* value, and the content is preferably 1.5 mM or less, more preferably 200 μM or less, from the viewpoint of the reaction efficiency. In this case, it is desired that the content of dNTP is 200 μM or more and 1 mM or less, respectively.

The content of a nucleotide analog in a reaction mixture for PCR is preferably 10 μM or more, more preferably 11 μM or more, from the viewpoint of sufficiently exhibiting an effect of lowering *Tm* value, and the content is preferably 1.5 mM or less, more

preferably 200 μM or less, from the viewpoint of the reaction efficiency. In this case, it is desired that the content of dNTP is 200 μM or more and 1 mM or less, respectively.

In the present specification, the term "a compound for lowering *Tm* value of a double-stranded nucleic acid" refers to a substance other than that incorporated in a nucleic acid as a substrate during cDNA synthesis and DNA amplification reaction, which acts to lower a dissociation temperature (*Tm* value) for denaturing a double-stranded nucleic acid into a single strand in the presence of the substance, the double-stranded nucleic acid including, for instance, a double-stranded DNA, an RNA-DNA double-stranded nucleic acid constituted by RNA and DNA, or the like. As such compounds, there can be used organic solvents such as formamide and dimethyl sulfoxide (DMSO) and amphoteric electrolytes (betains) represented by trimethyl glycine. In particular, formamide is preferably used in the present invention because it is said not to give mal-affects to the extension activity of a DNA polymerase. In addition, these compounds can be used in admixture of two or more kinds. For instance, favorable results can be obtained by using formamide and dimethyl sulfoxide in combination.

In addition, even if the nucleotide analog were a

single kind, there are some cases where there is no influence by the GC content depending on the kind of RNA used as a template. In such a case, more favorable results can be obtained by further using a compound for lowering *Tm* value of a double-stranded nucleic acid, and such an embodiment is also within the scope of the present invention.

The content of a compound for lowering *Tm* value of a double-stranded nucleic acid in a reverse transcription reaction mixture is preferably 20% by weight or less, more preferably 10% by weight or less, from the viewpoint of the reaction inhibition. In addition, during PCR reaction, the content of the compound in a reaction mixture for PCR is preferably 0.5% by weight or more, more preferably 1% by weight or more, from the viewpoint of lowering *Tm* value, and the content is preferably 20% by weight or less, more preferably 15% by weight or less, from the viewpoint of the reaction inhibition.

Concretely, when formamide is used, the content of formamide in a reverse transcription reaction mixture is preferably 20% by weight or less, more preferably 10% by weight or less, from the viewpoint of the reaction inhibition. In addition, the content of formamide in a reaction mixture for PCR is preferably 1% by weight or more, more preferably 1.5% by weight or more, from the

viewpoint of lowering *Tm* value, and the content is preferably 20% by weight or less, more preferably 15% by weight or less, from the viewpoint of the reaction inhibition.

Reverse transcription from RNA to cDNA is carried out in a reaction mixture comprising a sample containing a target RNA, a primer which is an oligonucleotide having a sequence complementary to the target, four kinds of deoxynucleotide triphosphates, an enzyme having a reverse transcription activity and at least one kind of a nucleotide analog. By this reaction, DNA having a nucleotide sequence complementary to the target RNA sequence mentioned above (cDNA) comprising a nucleotide analog can be synthesized. In addition, the resulting template RNA-cDNA double-stranded nucleic acid to be formed from cDNA and the target RNA has lower *Tm* value, as compared to that of a double-stranded nucleic acid without containing a nucleotide analog. For instance, there is exhibited an excellent property of dissociating into a single strand at a lower temperature, as compared to a usual double-stranded DNA without containing a nucleotide analog.

Next, a DNA fragment derived from cDNA can be amplified by carrying out the DNA amplification reaction, including, for instance, PCR, by the use of cDNA mentioned

above as a template. In this case, there can be selectively amplified a DNA fragment corresponding to cDNA, i.e. a DNA fragment corresponding to a target RNA, by carrying out DNA amplification reaction in the presence of a nucleotide analog under conditions that the template RNA-cDNA double-stranded nucleic acid mentioned above is denatured, and that a double-stranded DNA without containing a nucleotide analog admixed in a reaction mixture is not denatured, the conditions including, for instance, in PCR, setting a temperature for denaturation process. However, when various RNAs having different nucleotide sequences and chain lengths are used as targets, since the incorporation frequency of a nucleotide analog during the reverse transcription reaction varies depending on each target RNA, it may be difficult in some cases to set generalized conditions. In such a case, the conditions in which the selective amplification mentioned above takes place can be easily set by further adding to a DNA amplification reaction mixture a compound for lowering *Tm* value of a double-stranded nucleic acid as described above, including, for instance, formamide. For instance, when PCR is carried out by adding formamide to have a final concentration of 1% by weight to 20% by weight, only the amplified product corresponding to RNA can be obtained by setting a denaturation temperature of 80° to 85℃.

Concretely, in the embodiment of the method for amplifying a DNA of the present invention, the method is carried out in a DNA amplification reaction mixture in the presence of two or more kinds of nucleotide analogs or in the presence of a compound for lowering *Tm* value of a double-stranded nucleic acid. Particularly, an embodiment in which a nucleotide analog and a compound for lowering *Tm* value are coexistent is preferable. In this case, a nucleotide analog existing therein may be one or more kinds. A nucleotide analog is added to have the concentration mentioned above when a reaction mixture for PCR is prepared. When a reaction mixture for PCR is prepared by taking a part of the solution obtained after reverse transcription reaction, there may be used as a reaction mixture for PCR by adding only the components other than a nucleotide analog to the mixture, as long as a necessary amount of the nucleotide analogs is contained in the reverse transcription reaction mixture.

In the method of the present invention, cDNA synthesis reaction by the use of RNA as a template and DNA amplification reaction by the use of the cDNA as a template can be consecutively carried out using the same reaction mixture. In this case, a reaction mixture comprising both an enzyme having a reverse transcription activity and a DNA polymerase usable in PCR is used. Such

a reaction mixture can be prepared by a known method and used [*Bio Techniques* **18**, 678-687 (1995)]. Further, the same enzyme as that used in reverse transcription reaction can be also used for cDNA synthesis reaction and DNA amplification reaction by using a thermostable DNA polymerase having a reverse transcription activity.

The kit of the present invention is a kit usable for the above method, wherein the kit is capable of selectively amplifying DNA having a nucleotide sequence corresponding to RNA. Such a kit includes ones comprising one or more kinds of nucleotide analogs and a compound for lowering *Tm* value of a double-stranded nucleic acid, or ones comprising two or more kinds of nucleotide analogs or a compound for lowering *Tm* value of a double-stranded nucleic acid. Among them, the kit comprising two or more kinds of nucleotide analogs and a compound for lowering *Tm* value of a double-stranded nucleic acid is particularly preferable. The kit may comprise an enzyme having reverse transcriptase activity, a reaction buffer used in the enzyme reaction, a DNA polymerase usable in the DNA amplification reaction, and other reagents. In addition, the nucleotide analogs and the compound for lowering *Tm* value of a double-stranded nucleic acid mentioned above may be in a state of being added to a reaction buffer, or the like, to have an appropriate concentration upon use.

By using such a kit, it is made possible to easily carry out selective amplification of only the DNA fragment having a nucleotide sequence corresponding to RNA from the RNA sample admixed with DNA.

EXAMPLES

Next, the present invention will be described more concretely by means of the working examples, without intending to restrict the scope of the present invention to these working examples.

Example 1: Preparation of RNA and DNA

Each of RNAs and DNAs used in the following experiment was prepared respectively from human cultured cells HL-60 (ATCC CRL-1964) or human cultured cells HT-29 (ATCC HTB-38), respectively, using a TRIzol Reagent (manufactured by LIFE TECHNOLOGIES) in accordance with the procedures described in manual attached to the reagent. RNA derived from human cultured cells HL-60 had purity OD_{260}/OD_{280} of 1.8 or more, and DNA had purity OD_{260}/OD_{280} of 1.7 or more. In addition, RNA derived from human cultured cells HT-29 had purity OD_{260}/OD_{280} of 1.8 or more, and DNA had purity OD_{260}/OD_{280} of 1.7 or more.

Example 2: RT-PCR for various RNAs as Target

Selective amplification was tried with mRNAs having different chain lengths and GC contents as targets. There were selected as targets a region consisting of 621 bases (GC content: 38%) in transferrin receptor mRNA, a region consisting of 252 bases (GC content: 45%) in cytokeratin mRNA, and a region consisting of 275 bases (GC content: 58%) in β-actin mRNA. In the amplification of the transferrin receptor mRNA and the β-actin mRNA, RNA derived from human cultured cells HL-60 was used as a template, and in the amplification of the cytokeratin mRNA, RNA derived from human cultured cells HT-29 was used as a template, respectively.

As the primers for amplifying the above targets, primer P5 and primer P6 (SEQ ID NOs: 1 and 2 of Sequence Listing show the nucleotide sequences for each of primer P5 and primer P6) were synthesized for transferrin receptor; primer P1 and primer P2 (SEQ ID NOs: 3 and 4 of Sequence Listing show the nucleotide sequences for each of primer P1 and primer P2) were synthesized for cytokeratin; primer P7 and primer P8 (SEQ ID NOs: 5 and 6 of Sequence Listing show the nucleotide sequences for each of primer P7 and primer P8) were synthesized for β-actin, respectively.

Twenty microliters of a reverse transcription reaction mixture comprising 50 ng of RNA, or 50 ng of DNA,

and oligo dT adaptor primer attached to the kit was prepared by using RNA PCR Kit (AMV) Ver. 2.1 (manufactured by Takara Shuzo Co., Ltd.). The substrate nucleotides had seven kinds of compositions: One having the composition as described in the kit manual (Reaction Mixture 1); one in which dGTP was substituted by a mixture of dGTP and dITP (manufactured by Boehringer Mannheim) having each of the compositions shown in Table 1 (Reaction Mixtures 2 to 6, shown in Table 1); and one having the composition of Reaction Mixture 1 added with 62.5 μM each of 7-Deaza-dATP and 7-Deaza-dGTP (both manufactured by Boehringer Mannheim) (Reaction Mixture 7). Each of these reaction mixtures was set on PCR Thermal Cycler PERSONAL (manufactured by Takara Shuzo Co., Ltd.), and reverse transcription reaction was carried out at 50℃, 20 minutes - 5℃, 5 minutes.

Table 1

	dGTP	dITP
Reaction Mixture 2	1.75 mM	0.25 mM
Reaction Mixture 3	1.5 mM	0.5 mM
Reaction Mixture 4	1 mM	1 mM
Reaction Mixture 5	0.5 mM	1.5 mM
Reaction Mixture 6	0.25 mM	1.75 mM

The reaction mixture for PCR containing the primer pairs corresponding to each target mRNA was then prepared by using the above kit. Further, there were also prepared reaction mixtures by adding formamide to each of the reaction mixtures for PCR (the concentrations of formamide in each of the reaction mixtures for PCR being 6.25% by weight for transferrin receptor, 7.5% by weight for cytokeratin, and 13.75% by weight for β-actin). To the solution obtained after the reverse transcription reaction mentioned above was added 80 μl of these reaction mixtures, to make up a total volume of 100 μl of the reaction mixture. The resulting mixture was set on PCR Thermal Cycler PERSONAL, and 30 cycles of PCR were carried out, one cycle consisting of 82℃, 1 minute - 45℃, 1 minute - 72℃, 1 minute.

Eight microliters of the reaction mixture obtained as above was subjected to 3% agarose gel electrophoresis

using NuSieve 3:1 Agarose (manufactured by FMC). The amount of the product in each reaction mixture after termination of reaction was evaluated by subjecting the agarose gel after electrophoresis to ethidium bromide staining and comparing the fluorescence intensity emitted by ultraviolet ray irradiation. Of the obtained results, those in which RNA was added as a template in the reverse transcription reaction mixture are shown in Table 2.

Table 2

Target	Reverse Transcription Reaction Mixture	Formamide −	Formamide +
Transferrin Receptor	Reaction Mixture 1	−	++
	Reaction Mixture 2	−	+
	Reaction Mixture 3	−	++
	Reaction Mixture 4	+	++
	Reaction Mixture 5	+	+
	Reaction Mixture 6	−	±
	Reaction Mixture 7	−	++
Cytokeratin	Reaction Mixture 1	−	−
	Reaction Mixture 2	−	−
	Reaction Mixture 3	−	−
	Reaction Mixture 4	−	+
	Reaction Mixture 5	−	±
	Reaction Mixture 6	±	±
	Reaction Mixture 7	−	+
β-Actin	Reaction Mixture 1	−	++
	Reaction Mixture 2	−	±
	Reaction Mixture 3	−	++
	Reaction Mixture 4	−	++
	Reaction Mixture 5	−	±
	Reaction Mixture 6	−	−
	Reaction Mixture 7	−	++

−: No amplification was found.

±: Slight amplification was found.

+: Some amplification was found.

++: Strong amplification was found.

When PCR was carried out in a reaction system in which formamide was not added, only a slight amount of an amplified product was obtained in very limited reaction mixtures, whereas in a reaction system in which formamide was added, amplified products could be obtained for any of target RNAs by using Reaction Mixture 4 in the reverse transcription reaction. Further, in a reaction system in which formamide was added, amplified products could be obtained for all of the target RNAs by using Reaction Mixture 7 comprising two kinds of nucleotide analogs. In addition, when the target was cytokeratin, a desired amplified product could not be obtained when formamide was added during PCR without using a nucleotide analog. Incidentally, when DNA was added as a template to the reverse transcription reaction mixture, the amplified products could not be obtained, regardless of the compositions of the reaction mixtures.

It was shown from these results that various DNA fragments derived from RNAs can be efficiently amplified

under given temperature conditions when PCR was carried out by adding formamide during PCR and using as a nucleotide analog a given amount of dITP or a given amount of a combination of 7-Deaza-dATP and 7-Deaza-dGTP. In addition, since amplification does not take place under these conditions by the use of DNA as a template, it was also elucidated that DNA fragments by the use of RNA as a template were selectively amplified.

Example 3: Comparison of Combinations of Nucleotide Analogs

The following experiment was carried out with a region of about 2 kb in human transferrin receptor mRNA as a target.

Twenty microliters of a reverse transcription reaction mixture comprising 50 ng of RNA derived from human HL-60 cells, or 50 ng of DNA and the above primer P4 (SEQ ID NO: 7 of Sequence Listing shows the nucleotide sequence of primer P4), and the following nucleotide analogs, each at a concentration of 62.5 μM, was prepared by using RNA PCR Kit (AMV) Ver. 2.1.

Reaction Mixture 1: 7-Deaza-dGTP

Reaction Mixture 2: 7-Deaza-dATP

Reaction Mixture 3: dITP

Reaction Mixture 4: dITP, 7-Deaza-dATP

Reaction Mixture 5: 7-Deaza-dGTP, 7-Deaza-dATP

Each of these reaction mixtures was set on PCR Thermal Cycler PERSONAL, and the reverse transcription reaction was carried out under the conditions of 50℃, 20 minutes - 5℃, 5 minutes.

A reaction mixture for PCR comprising primer P4 and primer P9 (SEQ ID NO: 8 of Sequence Listing shows the nucleotide sequence of primer P9) was prepared by using RNA PCR Kit mentioned above. Eighty microliters of the reaction mixture was added to a solution obtained after the reverse transcription reaction as mentioned above to make up a total volume of the reaction mixture of 100 μl. The resulting mixture was set on PCR Thermal Cycler PERSONAL, and 30 cycles of PCR were carried out, one cycle consisting of 84℃, 1 minute - 60℃, 30 seconds - 72℃, 2 minutes.

Eight microliters of the resulting reaction mixture was subjected to 1% agarose gel electrophoresis prepared with Agarose L03 (manufactured by Takara Shuzo Co., Ltd.), and evaluated in the same manner as in Example 2. As a result, in all reaction conditions, only amplified fragments by the use of RNAs as templates were found, and the highest amplification efficiency was obtained when

Reaction Mixture 2 was used among reaction systems in which only one kind of the nucleotide analog was added. It was shown from the above results that the amplification efficiency differs depending upon the kinds of nucleotide analogs used. In addition, in Reaction Mixtures 4 and 5 using two kinds of nucleotide analogs, both reaction mixtures showed higher amplification efficiency, as compared to any of the reaction systems in which one kind of the nucleotide analog was added. In addition, in the reaction mixture to which DNA was added as a template, no amplified product was found regardless of the composition.

Example 4: RT-PCR Using Various Enzymes for Reverse Transcription Reaction

Twenty microliters of a reverse transcription reaction mixture comprising 500 ng of RNA or 250 ng of DNA, each prepared from human HL-60 cells, and primer P4, and added with 7-Deaza-dATP and 7-Deaza-dGTP, each at a final concentration of 62.5 μM was prepared by using RNA PCR Kit (AMV) Ver. 2.1 (AMV-RTase), BcaBEST RNA PCR Kit [*Bacillus caldotenax*-derived DNA polymerase (BcaBEST), manufactured by Takara Shuzo Co., Ltd.], or MMLV-RTase (manufactured by LIFE TECHNOLOGIES) in accordance with the manual for each of the enzymes. Each of these reaction mixtures was set on PCR Thermal Cycler PERSONAL, and the reverse

transcription reaction was carried out under the following program.

Program for Reverse Transcription Reaction

AMV RTase: 50℃, 20 minutes - 5℃, 5 minutes

BcaBEST: treating at 65℃, 1 minute - 30℃, 1 minute; thereafter, heating to 65℃ over a period of 15 minutes; subsequently 65℃, 15 minutes - 80℃, 2 minutes - 5℃, 5 minutes

MMLV-RTase: 42℃, 50 minutes - 70℃, 15 minutes - 5℃, 5 minutes

Subsequently, a reaction mixture for PCR was prepared by using the solution obtained after the reverse transcription reaction. The reaction mixture for PCR comprising primers P4 and P3 (SEQ ID NOs: 7 and 9 of Sequence Listing show the nucleotide sequences of each of primer P4 and primer P3), the reaction mixture being further added with formamide was prepared by using BcaBEST RNA PCR Kit when the reverse transcription reaction was carried out using BcaBEST, or using RNA PCR Kit (AMV) Ver. 2.1 when a reverse transcriptase other than BcaBEST was used, referring to the instruction manual for each kit. The concentration of formamide in the reaction mixture for

PCR was 2.5% by weight when BcaBEST RNA PCR Kit was used, or 6.25% by weight when RNA PCR Kit (AMV) Ver. 2.1 was used. Eighty microliters of each of these reaction mixtures for PCR was added to the solution after the reverse transcription reaction mentioned above to make up a total volume of 100 μl of a reaction mixture. The resulting reaction mixture was set on PCR Thermal Cycler PERSONAL, and 30 cycles of PCR were carried out, one cycle consisting of 82℃, 30 seconds - 60℃, 30 seconds - 72℃, 1 minute.

After the termination of reaction, 8 μl of the reaction mixture was taken and subjected to 1% agarose gel electrophoresis prepared with Agarose L03 (manufactured by Takara Shuzo Co., Ltd.), and evaluated in the same manner as in Example 2. As a result, regardless of the enzymes used, no amplified fragments were found in the reaction mixture in which DNA was added as a template. By contrast, amplified fragments of the same size (964 bp) were found in all of the reaction mixtures in which RNA was added.

Example 5: Application to RT-PCR by One Reaction Mixture

RT-PCR in which the reverse transcription reaction and PCR are carried out in one reaction mixture was carried out with a region consisting of 252 bases in human cytokeratin mRNA as a target.

By using GeneAmp EZ rTth RNA PCR Kit (manufactured by Perkin-Elmer), three kinds of reaction mixtures, specifically (1) one having the composition as described in the kit manual; (2) the composition added with dITP at a final concentration of 300 μM; and (3) the composition added with 7-Deaza-dATP and 7-Deaza-dGTP, at a final concentration of 150 μM each, and formamide at a final concentration of 2% by weight were prepared. To each of the reaction mixtures were added primer P1 and primer P2 mentioned above, 1 μg of RNA derived from human HT-29 cells, and 0, 50, 100 or 200 ng of DNA derived from the cells. For each of the resulting reaction mixtures, RT-PCR was carried out under two patterns of the following conditions using PCR Thermal Cycler PERSONAL.

Condition 1:

Reverse transcription reaction (60℃, 30 minutes)	once
Denaturation (94℃, 2 minutes)	once
PCR (94℃, 30 seconds - 60℃, 30 seconds - 72℃, 1 minute)	30 cycles

Condition 2:

Reverse transcription reaction (60℃, 30 minutes)	once

Denaturation (82℃, 2 minutes)	once
PCR (82℃, 30 seconds - 60℃, 30 seconds - 72℃, 1 minute)	30 cycles

Eight microliters of each of the reaction mixtures for PCR as obtained above was subjected to 3% agarose gel electrophoresis using NuSieve 3:1 Agarose, and the formed amplified product was analyzed. As a result, under Condition 1, regardless of the composition of the reaction mixtures, DNA-derived amplified product (604 bp) appeared as well as RNA-derived amplified product (251 bp), and its amplification level was dependent on the concentration of DNA added. Under Condition 2, although not any of the amplified products could be obtained in the reaction mixture (1), only amplified products in which RNA was used as a template were obtained in the reaction mixtures (2) and (3). In this case, as compared to the reaction mixture (2), the reaction mixture (3) showed higher amplification efficiency.

Example 6: RT-PCR Using Formamide and Dimethyl Sulfoxide in Combination

The effects to RT-PCR were compared between a case where only formamide was added to a reaction mixture, and a case where formamide and dimethyl sulfoxide were added

to a reaction mixture.

Each of the target mRNAs and the primers used for amplification were as follows: A region consisting of 275 bases (GC content: 58%) in β-actin mRNA: primer P7 and primer P8; a region consisting of 325 bases (GC content: 61%) in tumor necrosis factor (TNF) mRNA: primer P10 and primer P11 (SEQ ID NOs: 10 and 11 of Sequence Listing show the nucleotide sequences for each of primer P10 and primer P11); and a region consisting of 448 bases in Mcl mRNA (GC content: 51%), apoptosis-associated cytokine: primer P12 and primer P13 (SEQ ID NOs: 12 and 13 of Sequence Listing show the nucleotide sequences for each of primer P12 and primer P13).

By using One Step RNA PCR Kit (manufactured by Takara Shuzo Co., Ltd.), there was prepared a reaction mixture comprising 1 μg of RNA derived from human culture cells HL-60 and a primer corresponding to each target mentioned above and added with 100 μM each of 7-Deaza-dGTP and 7-Deaza-dATP according to the manual of the kit. Further, in addition to this reaction mixture, ones in which formamide was added to have a final concentration of 2, 4, 6, 8 or 10% by weight, respectively, to each reaction mixture, and one in which formamide was added to have a final concentration of 2% by weight and dimethyl sulfoxide was added to have a final concentration of 3% by weight to

the reaction mixture, i.e. seven reaction mixtures in total, were prepared against each of the targets. Each of the resulting reaction mixtures was set on PCR Thermal Cycler PERSONAL, and RT-PCR was carried out under the following conditions.

Reverse transcription reaction (50℃, 20 minutes)	once
Denaturation (85℃, 2 minutes)	once
PCR (85℃, 1 minute - 45℃, 1 minute - 72℃, 1 minute)	30 cycles

Eight microliters of each of the reaction mixtures obtained as above was subjected to 3% agarose gel elctrophoresis using NuSieve 3:1 Agarose, and the formed amplified product was analyzed in the same manner as in Example 2. The results thereof are shown in Table 3.

Table 3

Target		β-Actin	TNF	Mcl
No addition of organic solvent		−	−	−
Formamide	2% by weight	+	−	−
	4% by weight	+	±	+
	6% by weight	+	+	−
	8% by weight	±	−	−
	10% by weight	−	−	−
Formamide 2% by weight and Dimethyl Sulfoxide 3% by weight		++	++	++

−: No amplification was found.

±: Slight amplification was found.

+: Some amplification was found.

++: Strong amplification was found.

As shown in Table 3, it was demonstrated that while the concentration of formamide needs to be adjusted for each target RNA in order to obtain excellent amplification efficiency when using only formamide, any of the targets are efficiently amplified with a single reaction mixture when formamide was used in combination with dimethyl sulfoxide.

Example 7: Kit

As the kit usable for the method for amplifying DNA of the present invention, there was prepared the following kit for 20 reactions.

10 × Buffer for reverse transcription reaction 100 mM Tris-HCl (pH 8.3) 500 mM KCl 100 mM $MgCl_2$ 100 mM DTT 500 μM 7-Deaza-dGTP 500 μM 7-Deaza-dATP 10 mM dNTP	40 μl
AMV Reverse Transcriptase (5 U/μl)	20 μl
2 × Buffer for PCR Reaction 20 mM Tris-HCl (pH 8.3) 100 mM KCl 5% by weight Formamide 5% by weight Dimethyl Sulfoxide	800 μl
Taq DNA Polymerase (5 U/μl)	20 μl

INDUSTRIAL APPLICABILITY

According to the present invention, DNA having a nucleotide sequence derived from RNA can be selectively amplified. By using the method of the present invention, a DNA fragment derived from RNA can be amplified without previously purifying RNA in a sample, thereby imparting simplification of the experimental procedures and improvement in reproducibility.

CLAIMS

1. A method for amplifying a DNA by the use of a DNA fragment comprising a nucleotide analog as a template in the presence of nucleotide analogs, characterized in that the method for amplifying a DNA is carried out in the presence of two or more kinds of nucleotide analogs or in the presence of a compound for lowering *Tm* value of a double-stranded nucleic acid.

2. A method for amplifying a DNA by the use of a DNA fragment comprising a nucleotide analog as a template in the presence of nucleotide analogs, characterized in that the method for amplifying a DNA is carried out in the presence of one or more kinds of nucleotide analogs and a compound for lowering *Tm* value of a double-stranded nucleic acid.

3. The method for amplifying a DNA according to claim 1 or 2, characterized in that amplification of a DNA is carried out by polymerase chain reaction.

4. The method for amplifying a DNA according to any one of claims 1 to 3, characterized in that the DNA fragment is a cDNA prepared by reverse transcription reaction using

an RNA as a template in the presence of nucleotide analogs.

5. The method for amplifying a DNA according to claim 4, characterized in that the DNA fragment is a cDNA prepared by reverse transcription reaction using an RNA as a template in the presence of two or more nucleotide analogs.

6. The method for amplifying a DNA according to any one of claims 1 to 5, characterized by the use, as the nucleotide analog, of a nucleotide analog having a property of lowering *Tm* value of a double-stranded nucleic acid to which the nucleotide analog is to be incorporated.

7. The method for amplifying a DNA according to any one of claims 1 to 6, characterized by the use of a nucleotide analog to be incorporated in a DNA strand in place of dATP or dTTP, and the use of a nucleotide analog to be incorporated in a DNA strand in place of dCTP or dGTP.

8. The method for amplifying a DNA according to any one of claims 1 to 7, wherein the nucleotide analog is selected from the group consisting of 7-Deaza-dGTP, 7-Deaza-dATP, dITP and hydroxymethyl dUTP.

9. The method for amplifying a DNA according to any one

of claims 1 to 8, characterized in that one or more kinds of compounds selected from the group consisting of formamide, dimethyl sulfoxide and trimethyl glycine are used as the compound for lowering *Tm* value of a double-stranded nucleic acid.

10. A kit for amplifying a DNA in the presence of a nucleotide analog by the use of a DNA fragment comprising a nucleotide analog as a template, characterized in that the kit comprises two or more kinds of nucleotide analogs or a compound for lowering *Tm* value of a double-stranded nucleic acid.

11. A kit for amplifying a DNA in the presence of a nucleotide analog by the use of a DNA fragment comprising a nucleotide analog as a template, characterized in that the kit comprises one or more kinds of nucleotide analogs and a compound for lowering *Tm* value of a double-stranded nucleic acid.

12. The kit according to claim 10 or 11, characterized in that the kit comprises a reagent for synthesizing a cDNA which is complementary to an RNA in the presence of nucleotide analogs.

13. The kit according to any one of claims 10 to 12, characterized in that the kit comprises as the nucleotide analog a nucleotide analog having a property of lowering *Tm* value of a double-stranded nucleic acid to which the nucleotide analog is to be incorporated.

14. The kit according to any one of claims 10 to 13, characterized in that the kit comprises a nucleotide analog to be incorporated in a DNA strand in place of dATP or dTTP, and a nucleotide analog to be incorporated in a DNA strand in place of dCTP or dGTP.

15. The kit according to any one of claims 10 to 14, wherein the nucleotide analog is selected from the group consisting of 7-Deaza-dGTP, 7-Deaza-dATP, dITP and hydroxymethyl dUTP.

16. The kit according to any one of claims 10 to 15, characterized in that the kit comprises, as the compound for lowering *Tm* value of a double-stranded nucleic acid, one or more kinds of compounds selected from the group consisting of formamide, dimethyl sulfoxide and trimethyl glycine.

ABSTRACT

A method for amplifying a DNA by the use of a DNA fragment comprising a nucleotide analog as a template in the presence of nucleotide analogs, characterized in that the method for amplifying a DNA is carried out in the presence of two or more kinds of nucleotide analogs or in the presence of a compound for lowering *Tm* value of a double-stranded nucleic acid, or characterized in that the method for amplifying a DNA is carried out in the presence of one or more kinds of nucleotide analogs and a compound for lowering *Tm* value of a double-stranded nucleic acid; and a kit for amplifying a DNA in the presence of a nucleotide analog by the use of a DNA fragment comprising a nucleotide analog as a template, characterized in that the kit comprises two or more kinds of nucleotide analogs or a compound for lowering *Tm* value of a double-stranded nucleic acid, or characterized in that the kit comprises one or more kinds of nucleotide analogs and a compound for lowering *Tm* value of a double-stranded nucleic acid. According to the present invention, a DNA fragment derived from RNA can be amplified without previously purifying RNA in a sample, thereby imparting simplification of the experimental procedures and improvement in reproducibility.

BIRCH, STEWART, KOLASCH & BIRCH, LLP

P.O. Box 747 • Falls Church, Virginia 22040-0747

Telephone: (703) 205-8000 • Facsimile: (703) 205-8050

ATTORNEY DOCKET NO.
1422-411P

PLEASE NOTE: YOU MUST COMPLETE THE FOLLOWING: ↓

COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT AND DESIGN APPLICATIONS

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor (if only one inventor is named below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Insert Title: →

METHODS FOR DNA AMPLIFICATION AND KITS THEREFOR

Fill in Appropriate Information — For Use Without Specification Attached: →

the specification of which is attached hereto. If not attached hereto,

the specification was filed on _____ as United States Application Number _____; and amended on _____ (if applicable); and/or the specification was filed on August 10, 1998 as PCT International Application Number PCT/JP98/03566; and was amended under PCT Article 19 on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Insert Priority Information: → (if appropriate)

Prior Foreign Application(s)			Priority Claimed	
9-231885 (Number)	Japan (Country)	August 14, 1997 (Month / Day / Year Filed)	☒ Yes	☐ No
9-305016 (Number)	Japan (Country)	October 21, 1997 (Month / Day / Year Filed)	☒ Yes	☐ No
(Number)	(Country)	(Month / Day / Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Month / Day / Year Filed)	☐ Yes	☐ No

Insert Provisional Application(s): → (if any)

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

(Application Number) (Filing Date)

(Application Number) (Filing Date)

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More than 12 Months (6 Months for Designs) Prior to the Filing Date of This Application:

Insert Requested Information: → (if appropriate)

Country	Application Number	Date of Filing (Month / Day / Year)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States and/or PCT application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States and/or PCT application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Insert Prior U.S. Application(s): → (if any)

(Application Number)	(Filing Date)	(Status — patented, pending, abandoned)
(Application Number)	(Filing Date)	(Status — patented, pending, abandoned)

I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

Raymond C. Stewart	(Reg. No. 21,066)	Terrell C. Birch	(Reg. No. 19,382)
Joseph A. Kolasch	(Reg. No. 22,463)	James M. Slattery	(Reg. No. 28,380)
Bernard L. Sweeney	(Reg. No. 24,448)	Michael K Mutter	(Reg. No. 29,680)
Charles Gorenstein	(Reg. No. 29,271)	Gerald M. Murphy, Jr.	(Reg. No. 28,977)
Leonard R. Svensson	(Reg. No. 30,330)	Terry L. Clark	(Reg. No. 32,644)
Andrew D. Meikle	(Reg. No. 32,868)	Marc S. Weiner	(Reg. No. 32,181)
Joe McKinney Muncy	(Reg. No. 32,334)	C. Joseph Faraci	(Reg. No. 32,350)
Donald J. Daley	(Reg. No. 34,313)	John W. Bailey	(Reg. No. 32,881)
John A. Castellano	(Reg. No. 35,094)		

17

Send Correspondence to: BIRCH, STEWART, KOLASCH & BIRCH, LLP
P.O. Box 747 • Falls Church, Virginia 22040-0747
Telephone: (703) 205-8000 • Facsimile: (703) 205-8050

PLEASE NOTE: YOU MUST COMPLETE THE FOLLOWING: ↓

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1.00

Full Name of First or Sole Inventor: Insert Name of Inventor → Insert Date This Document is Signed

GIVEN NAME	FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
Junko	YAMAMOTO	Junko Yamamoto	2000, 1, 20

Insert Residence → Insert Citizenship

Residence (City, State & Country)	CITIZENSHIP
Otsu-shi, Shiga 520-0831 Japan JPX	Japan

Insert Post Office Address →

POST OFFICE ADDRESS (Complete Street Address including City, State & Country)
17-10-405, Matsubara-cho, Otsu-shi, Shiga 520-0831 Japan

Full Name of Second Inventor, if any: see above

2.00

GIVEN NAME	FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
Hiroyuki	MUKAI	Hiroyuki Mukai	2000, 1. 20

Residence (City, State & Country)	CITIZENSHIP
Moriyama-shi, Shiga 524-0102 Japan JPX	Japan

POST OFFICE ADDRESS (Complete Street Address including City, State & Country)
1461-82, Aza Minamikawa, Mizuho-cho, Moriyama-shi, Shiga 524-0102 Japan JPX

Full Name of Third Inventor, if any see above

3.00

GIVEN NAME	FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
Fumitsugu	HINO	Fumitsugu Hino	2000. 1. 13

Residence (City, State & Country)	CITIZENSHIP
Kusatsu-shi, Shiga 525-0028 Japan JPX	Japan

POST OFFICE ADDRESS (Complete Street Address including City, State & Country)
3-9-8, Kamigasa, Kusatsu-shi, Shiga 525-0028 Japan

Full Name of Fourth Inventor, if any see above

4.00

GIVEN NAME	FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
Ikunoshin	KATO	Ikunoshin Kato	2000. 1. 18

Residence (City, State & Country)	CITIZENSHIP
Uji-shi, Kyoto 611-0028 Japan	Japan

POST OFFICE ADDRESS (Complete Street Address including City, State & Country)
1-1-150, Nanryo-cho, Uji-shi, Kyoto 611-0028 Japan

Full Name of Fifth Inventor, if any see above

GIVEN NAME	FAMILY NAME	INVENTOR'S SIGNATURE	DATE*

Residence (City, State & Country)	CITIZENSHIP

POST OFFICE ADDRESS (Complete Street Address including City, State & Country)

* DATE OF SIGNATURE

SEQUENCE LISTING

<110 > Takara Shuzo Co., Ltd.

<120 > A DNA amplification method and a kit therefor

<130 > 98-031-PCT

<150 > JP 9/231885

<151 > 1997-8-14

<150 > JP 9/305016

<151 > 1997-10-21

<160 > 13

<210 > 1

<211 > 20

<212 > DNA

<213 > Artificial Sequence

<400 > 1

ccacagtgtc tgtatcggag 20

<210 > 2

<211 > 20

<212 > DNA

<213 > Artificial Sequence

<400 > 2

gtttccaact gccctatgac 20

<210 > 3

<211 > 22

<212 > DNA

<213 > Artificial Sequence

<400 > 3

cgtctaacag tggaagctga tc 22

<210 > 4

<211 > 22

<212 > DNA

<213 > Artificial Sequence

<400 > 4

catgacttca tacttctgcc tc 22

<210 > 5

<211 > 21

<212 > DNA

<213 > Artificial Sequence

<400 > 5

caagagatgg ccacggctgc t 21

<210 > 6

<211 > 21

<212 > DNA

<213 > Artificial Sequence

<400 > 6

tccttctgca tcctgtcggc a 21

<210 > 7

<211 > 20

<212 > DNA

<213 > Artificial Sequence

<400 > 7

gagactgtga gtagtgacac 20

<210 > 8

<211 > 20

<212 > DNA

<213 > Artificial Sequence

<400 > 8

ccatcccatc atcttggtac 20

<210 > 9

<211 > 20

<212 > DNA

<213 > Artificial Sequence

<400 > 9

ctaagaagcg agcactgacc 20

<210 > 10

<211 > 20

<212 > DNA

<213 > Artificial Sequence

<400 > 10

cagagggaag agttccccag 20

<210 > 11

<211 > 20

<212 > DNA

<213 > Artificial Sequence

<400 > 11

ccttggtctg gtaggagacg 20

<210 > 12

<211 > 21

<212 > DNA

<213 > Artificial Sequence

<400 > 12

cggcagtcgc tggagattat c 21

<210 > 13

<211 > 21

<212 > DNA

<213 > Artificial Sequence

<400 > 13

tctaggtcct ctacatggaa g 21